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Photosystem II (PSII) is the membrane-bound protein complex which participates in the photosynthetic conversion of sunlight energy into chemical energy and produces molecular oxygen. Calcium and chloride are important cofactors that PSII requires in the process of oxygen evolution. They may be thought of as involved in a bisubstrate mechanism, where each is required for the activation of evolution of oxygen. The binding site of calcium has been identified as part of the catalytic Mn_4Ca cluster, and two chloride binding sites have been identified near the catalytic cluster.

In this research, the dependence of oxygen evolution activity by PSII as a function of chloride and calcium concentrations was measured and analyzed according to enzyme kinetics methods to determine a series of K_M values for Cl^- activation. The data were next analyzed in terms of bisubstrate enzyme kinetics models, leading to kinetic constants for both Ca^{2+} and Cl^- activation. While the order of binding could not be determined from the data, the results are consistent with a random sequential binding model for the two ions. Further experiments were performed to reveal details about the calcium and chloride binding sites. The possibility of using isothermal titration calorimetry (ITC) to analyze Cl^- binding to PSII was explored.

Finally, the response of the Tyr Z radical, which is involved in electron transfer from the Mn_4Ca cluster, was analyzed using electron paramagnetic resonance (EPR) spectroscopy, to determine the effects of the presence of Ca^{2+} and Cl^-. The results of these studies help to clarify the relationship between the critically required activators calcium and chloride during oxygen evolution by PSII.

CALCIUM AND CHLORIDE AS COFACTORS

AND THEIR BINDING IN

PHOTOSYSTEM II

by

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APPROVAL PAGE

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LIST OF ABBREVIATIONS

PSI	Photosystem I
PSII	Photosystem II
OEC	Oxygen Evolution Complex
MES	2-(N-morpholino) ethanesulfonic acid
ITC	Isothermal Titration Calorimetry
EPR	Electron Paramagnetic Resonance
NMR	Nuclear Magnetic Resonance
Tyr	Tyrosine

CHAPTER I

INTRODUCTION

Thesis Goals:

Photosystem II (PSII) is the membrane bound protein complex which participates in converting the sunlight into chemical energy. Calcium ion and chloride are among the different cofactors that PSII requires in the process of photosynthesis. They are involved in a bisubstrate enzyme activity, where each of them is required for the evolution of oxygen. The binding site of calcium ion has been identified as part of the catalytic site, and the two binding sites for chloride have been identified to be near the site of oxygen evolution. The goal of this research was to better understand the calcium and chloride requirements by PSII. The main objectives were to:

i) Measure the dependence of oxygen evolution activity by PSII as a function of chloride and calcium ion concentrations;

ii) Analyze the activity data in terms of bisubstrate enzyme kinetics involved in binding of Ca^{2+} and Cl^- ions in PSII;

iii) Determine if it is possible to use isothermal titration calorimetry (ITC) for studying calcium ion and chloride binding in PSII;

iv) Analyze the response of the Tyr Z radical for the presence of Ca^{2+} and Cl^- using electron paramagnetic resonance (EPR) spectroscopy.

Photosynthesis:

Photosynthesis involves the conversion of light energy into chemical energy, which is catalytically achieved by Photosystem II (PSII) and Photosystem I (PSI). The protein complexes PSI and PSII are embedded in the thylakoid membranes, within chloroplasts.[1] Water splitting at the Mn_4Ca cluster of PSII provides electrons to the electron transport chain which results in an electro-chemical gradient across the membrane ultimately leading to the production of organic carbon compounds.[2,3,4]

$$\text{Sunlight} + 6H_20 + 6CO_2 \rightarrow 60_2 + C_6H_{12}O_6$$

Photosynthesis is divided into 2 phases, the light phase and the dark phase, with the light phase taking place in PSII and PSI and the dark phase taking place in the stroma (the fluid between the thylakoid membranes) ending in the production of sugar molecules. The light reactions are often described by the Z Scheme of photosynthesis (Figure 1).

The Z Scheme:

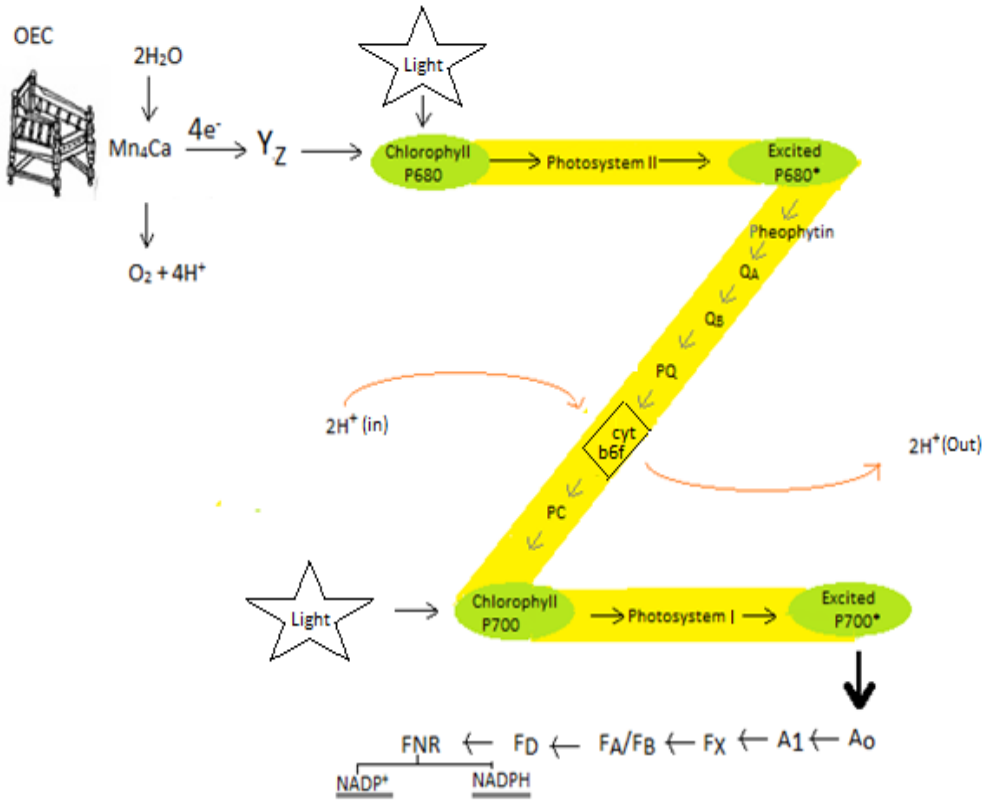

Figure 1. The Z Scheme of Photosynthesis, Based on Govindjee's Illustration[1]. It shows the synchronized activity between the PSI and the PSII complexes which results in formation of NADPH and the transmembrane proton gradient used for energy transduction. Key: OEC - oxygen evolving complex; Y_Z - tyrosine Z; Q_A, Q_B, PQ - plastoquinones; PC - plastocyanin; A_0 - chlorophyll acceptor; A_1 - phylloquinone; F_X, F_A, F_B - iron-sulfur clusters; FD - ferredoxin; FNR - ferredoxin-$NADP^+$ reductase; NADPH - nicotinamide adenine dinucleotide phosphate.

Photosystem II:

The core of PSII is about 350 kDa in molecular weight and composed of 17 or more transmembrane subunits, several peripheral proteins, and many cofactors. In PSII from all known species, the peripheral subunit PsbO stabilizes the catalytic Mn cluster. In higher plant PSII, the peripheral subunits PsbP and PsbQ, which are 23 and 17 kDa in weight, respectively, regulate the access of the inorganic ions Ca^{2+} and Cl^- to the site of oxygen evolution. PSII provides electrons from water to the electron transport chain, evolving oxygen as a side product with the help of the Mn cluster. This Mn_4Ca complex provides the site where water molecules are broken down as the complex cycles through different Mn oxidation states, known as the S states.[4]

During photosynthesis the sunlight captured by the PSII antenna chlorophyll is passed onto P680, the reaction center where charge separation occurs. This leads to the excited state of P680 which is denoted by P680*. The reaction center P680 is so called due to its absorption maximum at 680 nm. It is a multimer of weakly coupled chlorophyll with different excited states. P680* attains an oxidized state, denoted by $P680^+$, after passing an electron to pheophytin. $P680^+$ is known to be one of the most oxidizing species among the living organisms, hence providing it with the ability of withdrawing an electron from the water molecule.[5,6]

For $P680^+$ to return to its reduced state, P680, it accepts an electron from the redox active tyrosine residue Y_Z, which is Tyr 161 of the D1 subunit. Y_Z then accepts an electron from the oxygen evolving complex (OEC), which contains the Mn_4Ca cluster where the water splitting occurs.

Meanwhile, the electron that was transferred to pheophytin is then transferred to two plastoquinones (Q_A and Q_B) which are derivatives of *p*-benzoquinone. The first plastoquinone Q_A is covalently bound to PSII, while Q_B is loosely bound and can leave PSII once it is reduced to plastoquinol. Q_B accepts two protons from the matrix of the stroma along with two electrons from Q_A. The electrons are later transferred to the cytochrome b_6f protein complex whereas the protons are transferred to the lumen or interior of the thylakoid membrane.

XRD Structure of Photosystem II:

There has been extensive research carried out to understand the structure of PSII, since it is within this structure that the important components of photosynthesis are present. In recent years, some scientists had ground breaking X-ray crystallography results that contributed to the explanation of the structure. All structures thus far solved have been from thermophilic cyanobacteria, *Thermosynechococcus elongatus* or *T. vulcanus*.

The first X ray diffraction (XRD) structure with an active manganese cluster had a resolution of 3.8 Å and was presented in 2001 by Zouni, Kern and group at German universities[7]. The structure presented the organization of cofactors and subunits, and revealed the position and approximate shape of the Mn_4Ca cluster. In 2009 a paper was presented by Guskov and group with the structure of PSII at a resolution of 2.9 Å[8]. According to the structure each monomeric unit of the dimeric structure had 20 protein subunits, 35 chlorophyll a molecules, 12 carotenoids, 25

integral lipids, calcium ions and a chloride ion.[8] A more recent structure was elucidated by Umena and his group at a resolution of 1.9 Å, illustrated in Figure 2.[9] At this resolution, water molecules can be seen.

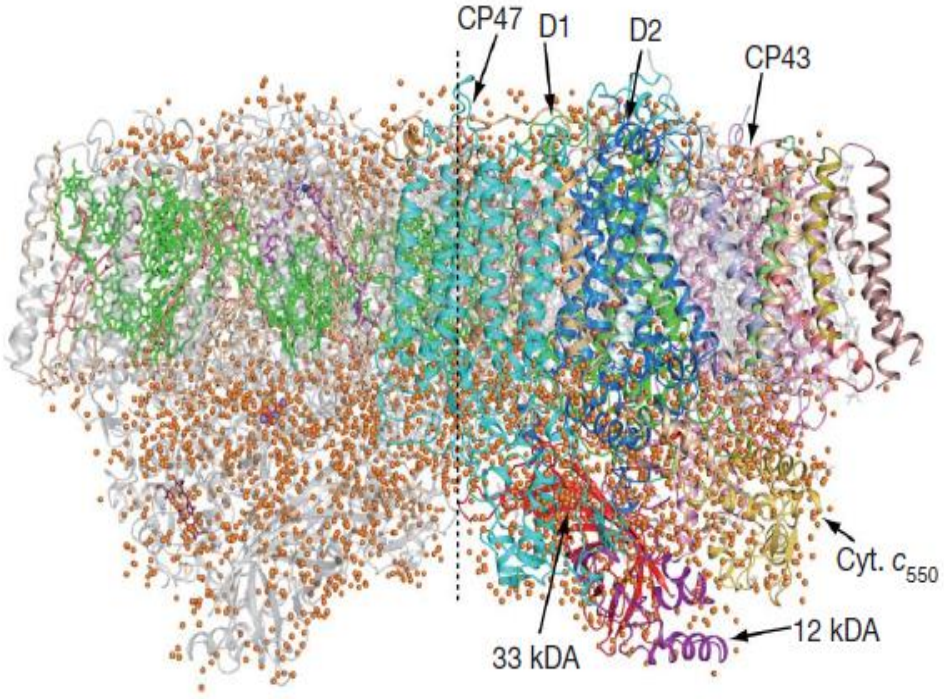

Figure 2. The Structure of PSII as Illustrated at 1.9 Å from *T. Vulcanus* by Umena[9]. The structure shows the protein subunits in the monomer with the cofactors and the water molecules are represented in orange color.

The integral membrane part of PSII has 36 transmembrane alpha helices, 22 of which are assigned to subunits D1, D2, CP43 and CP47. These are arranged in twofold pseudorotation symmetry. The D1 and D2 subunits each have five transmembrane helices, A through E, which are arranged as interlocking semicircles related by a pseudoaxis. The subunits D1 and D2 are distinguished clearly due to the presence of the Mn cluster, which the D1 subunit coordinates. The Mn cluster is present towards the luminal side near a short alpha helix between helices C and D. The Q_A binding site is present towards the stromal side on the D2 subunit.

The charge separating complex P680 consists of 4 chlorophyll a molecules. Nearby are two pheophytin molecules, which are chlorophylls without the central Mg^{2+} ion. Pheophytin receives an electron when initial charge separation occurs, then passes it on to Q_A.

The chlorophyll containing proteins CP43 and CP47 both act as antenna proteins and are involved in light harvesting. CP43 and CP47, with 48 and 52 kDa molecular weights, respectively, are situated next to D1 and D2 subunits. CP43 and CP47 also contribute to the protein environment for the water splitting apparatus.

In the recent paper by Umena and group, which presented the XRD structure of PSII at an excellent resolution of 1.9 Å, as many as five oxygen atoms were found bound as oxo bridges within the penta metallic Mn_4Ca cluster.[9] It was thought that one of them acts as substrate for the atmospheric oxygen produced as the end product. In addition, around 1300 water molecules were found in each monomer of PSII, indicating a huge number of hydrogen bonds.

S States:

During oxygen evolution the OEC undergoes 5 intermediate states denoted by S_i where i = 0 – 4, with the higher number corresponding to the higher oxidation state.[5,6] These states were used to explain the results of flash studies in which oxygen yield was observed in response to brief, carefully spaced flashes of light. The most stable S–State is the S_1 state, which occurs in dark-adapted cells or PSII samples. S_0 was also found to be relatively stable, whereas S_2 and S_3 were found to relax to the lower S-states, with a halftime of 200 μs or more. The S_4 state is very unstable and reduces to the S_0 state almost immediately with simultaneous evolution of O_2.

Several electron paramagnetic resonance (EPR) studies have been carried out to understand the Mn oxidation states in the S–state cycle. One signal that has been frequently studied is a multiline signal (MLS) that was found to be associated with the S_2 state. Starting from dark-adapted samples, the maximum MLS was observed on the first flash, indicating that the signal arose from the S_2 state. The characteristics of the MLS indicated that it arose from a mixed valence Mn cluster that probably included Mn(III) and Mn(IV), which helped in assigning the oxidation states to Mn in the S–State cycle. [10,11]

The following average oxidation states were assigned to Mn in the S State cycle based on the above studies, as well as many other studies using EPR spectroscopy and X-ray absorption near edge structure (XANES). The number following the Mn corresponds to the numbering within the structure.

Table 1. Various S States with Average Oxidation States for Mn

S State	Mn(1)	Mn(2)	Mn(3)	Mn(4)
S_0	II	III	IV	IV
or	III	III	III	IV
S_1	III	III	IV	IV
S_2	III	IV	IV	IV
S_3	III	IV	IV	IV* (excited state)
or	IV	IV	IV	IV

The change in the XANES for the transition of S_2 to S_3 was very low, which led to a suggestion that there might have been no Mn oxidation, but oxidation of a nearby residue instead.[11]

Almost no studies have been able to detect the presence of the S_4 state in the cycle. However, a few spectroscopic, X–ray and EPR studies carried out indicate a lag in the transition from S_3 to S_0, suggesting the presence of an S_4 state.[12]

Oxygen Evolving Complex:

The OEC, where water oxidation takes place, consists of the Mn_4Ca cluster and the various ions, cofactors, and residues that contribute to water oxidation. The OEC contains a cubane like structure of Mn_3CaO_4 cluster, with three μ-oxo bridges formed by each of the Mn ions and the Ca ion, linked to a fourth Mn ion by μ-oxo bridges. The OEC is located close to the surface of the D1 subunit in an extrinsic

domain. The Mn cluster is at a distance of 18.5 Å from the center of the P680 chlorophyll cluster.[13,14]

In the 2011 XRD structure by Umena and coworkers, Figure 3,[9] the Mn ions within the Mn_3CaO_4 cubane are not placed at the vertices of an exact cube, but have inter-Mn distances ranging from 2.8 – 3.3 Å. The fourth Mn has a distance of 5.0 – 5.4 Å from the two farthest Mn. The distance of the calcium ion from the Mn ions ranges from 3.3 – 3.8 Å, with its position at one of the corners of the cubane structure. The other four corners of the cubane structure are occupied by the oxygen atoms. The bond length of the Mn-O bonds is around 1.8 – 2.1 Å and the bond length of Ca^{2+} – O is around 2.4 – 2.5 Å. The fourth Mn which is outside the cubane is connected with the two Mn ions through μ-oxo bridges including a fifth oxygen. The whole structure resembles a distorted chair.

There are four water molecules resolved in the first coordination sphere, two of which are associated with the fourth Mn and the other two with the calcium ion. This discovery led to the speculation that the water molecules present in these sites might act as substrate in the photosynthetic reaction. The water molecules are placed at 2.1 Å and 2.2 Å from the Mn atom and 2.4 Å from the Ca^{2+} ion. None of the other three Mn ions are associated with any of the water molecules.[15]

Different proposals have been put forward for the mechanism of O_2 formation with a few suggesting the involvement of μ-oxo bridges with Mn, a few suggesting the oxyl radical and a few suggesting the role of calcium bound water molecules. These suggestions include a proposed nucleophilic attack on

electrophilic Mn-O bonds.[16] Research shows that the O_2 bond is formed during the S_4 state.[15,17]

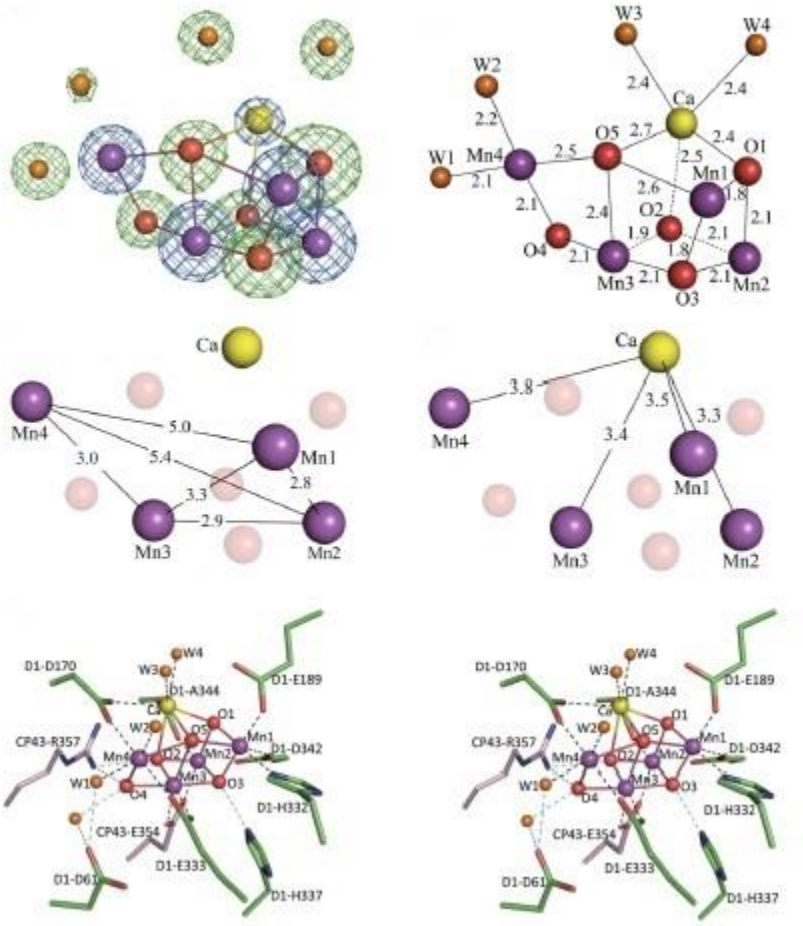

Figure 3. The Structure of the Mn_4CaO_5 Cluster as Illustrated by Umena[9]. The above figure presents the various atoms associated with the Mn cluster and the distances between the atoms in PSII.

Calcium Binding Sites:

Calcium has an important role in water splitting as hypothesized by many scientists. Calcium acts along with chloride as cofactor in water oxidation. Occupying a spot in one of the corners of the near cubane structure, it is placed at a distance of 3.5, 3.3, 3.4 and 3.8 Å from each Mn atom. As discussed earlier calcium is bound to two water molecules, one of which is bound to a Tyr Z residue in the D1 subunit. Calcium is retained in its position with the help of the subunits PsbP and PsbQ, with molecular weights 23 and 17 kDa respectively. However, experiments conducted by Wincencjusz *et al.* in 1997 showed that the subunits could be depleted at low concentrations of Na_2SO_4 at pH 7.5 without removing the calcium ion. This proved that calcium was directly bound through other subunits and cofactors.[18]

Calcium can be depleted from PSII through the most commonly used methods. Wash procedures at a pH around 6.3 with 1 to 2 M NaCl produces PSII complexes depleted of the PsbP and PsbQ subunits. A part of the calcium ions is also removed from the Mn cluster. This brings down the activity by about 50-70% in the absence of Ca^{2+}. [19, 20, 21]

The Ca^{2+}- independent activity that remains after high NaCl wash indicates that some Ca^{2+} remains behind. In an effort to further remove Ca^{2+}, there have been certain modifications done to the NaCl wash procedures.[22] Exposure to actinic light in the presence of EDTA buffer helped further bring down the photosynthetic activity. As experiments were conducted it was concluded that the photosynthetic activity could be restored by the addition of both calcium and chloride.

According to the structures of the Mn cluster the presence of one calcium atom at the OEC was established.[13] However other binding sites for Ca^{2+} probable exist. For example, studies carried out in 1993 by Han *et al.*[23] discussed the presence of calcium ions in the antenna subunits. Experiments further conducted with NaCl-washed PSII showed a stoichiometry of 2-3 Ca per PSII. Later the experiments of Adelroth in 1995 using $^{45}Ca^{2+}$ confirmed that there is only one high affinity binding site for calcium in the OEC. [20] This leads to the conclusion that there are probably other binding sites in the protein complex but not in the OEC.

Experiments were also conducted by different research groups to understand the competitive binding of various metals with calcium. Trivalent metal ions were found to be more competitive than the divalent Sr^{2+} or monovalent Na^{+} ions.[24, 25, 26] These include lanthanides at lower concentrations of 50 – 100 μM, however at higher concentrations they caused irreversible damage to the PSII.

Chloride Binding Sites:

Chloride has been found to play a very important role in the O_2 evolution activity of PSII. Activation by chloride has been documented in several other enzymes. A well-known example is α–amylases [27], where chloride ions have important supportive activity.

Similar to calcium ions, chloride ions are also bound tightly with the help of PsbP and PsbQ subunits. Chloride affinity is higher in the presence of these subunits but it decreases upon their removal. Researchers have found using $^{36}Cl^{-}$ that there

is one high affinity chloride binding site within the OEC [28], but two sites are observed near the Mn cluster in XRD studies.[8,9] In activation experiments with PSII, the amount of oxygen evolved during photosynthesis increases with increasing chloride concentration, until reaching a maximum at saturation around 25 mM. Research shows that some other anions can promote oxygen evolution activity upon replacing chloride ions. Bromide promotes the photosynthetic activity of PSII to the same level as chloride.[29] Iodide activates in place of chloride at lower concentrations however at higher concentrations it plays an inhibitory role.[30] Nitrate ion activates as does bromide however with a lower turnover number.

Bromide's ability to replace chloride and retain maximum of the photosynthetic activity has been exploited by scientists to understand the location of chloride binding.[8, 9, 29] XRD studies had been unsuccessful in locating chloride in the PSII structure. Since bromide has the chemical property of absorbing at higher energies than chloride, crystallographic studies were carried out using PSII in which chloride had been replaced with bromide. These studies reveal a distance of 6-7 Å for the two chloride/bromide binding sites from the Mn cluster. A distance of 12 – 15 Å from the Y_Z molecule was also found, placing the chloride too far to have a direct effect on electron transfer to Y_Z.

A hypothesis has been presented by Pokhrel and group suggesting that the chloride ion may be involved in breaking a salt bridge between the D1 and D2 subunits. According to this hypothesis if the chloride ion is absent, there is an electrostatic attraction between the D1 and D2 subunits creating differences in the

structure of the Mn cluster and OEC, thus creating a difference in the ground spin state. This happens since the Cl^- binds to ligands associated with both D1 and D2 subunits, with one ligand coming from the D1 subunit and the other from the D2 subunit.

Overview of Enzyme Kinetics:

For the following equilibrium in which enzyme E binds substrate S to form product P,

$$E + S \underset{k_{-S}}{\overset{k_S}{\rightleftharpoons}} ES \xrightarrow{k_p} E + P$$

The reaction velocity, v, is given by the equation (Henri-Michaelis-Menten equation)

$$v = \frac{V_{max}[S]}{K_M + [S]}$$

where V_{max} is the maximum velocity and K_M is the Michaelis constant, given by

$$V_{max} = k_p[E]_t$$

$$K_M = \frac{k_{-S}+k_p}{k_S}$$

A plot of 1/v versus 1/[S] is known as a Lineweaver-Burk plot and has y-intercept corresponding to $1/V_{max}$ and slope corresponding to K_M/V_{max}.

In some cases, more than one substrate participates in a reaction leading to products. For the experiments presented in this thesis, bisubstrate activity was observed for the calcium and chloride ions that activate oxygen evolution. Although not strictly substrates for O_2 formation, the activators calcium and chloride can be treated as substrates in the enzyme kinetics analysis.

In a bisubstrate enzyme catalyzed reaction in which both the substrates bind to the enzyme before the products are formed, the binding is called sequential. (For bisubstate enzyme kinetics, see reference [31]). Sequential binding is expected for the activation of the OEC by calcium and chloride, since only a single product, oxygen, is formed. This is in contrast to a reaction in which there are two products, one released after the binding of the first substrate and the other after the binding of the second substrate.

Sequential bisubstrate reactions are further divided according to whether the binding is ordered or random. In an ordered sequential reaction, the substrates must bind to their sites in a specific order. However, in a random sequential reaction, the substrates can bind in either order.

Random Sequential Binding:

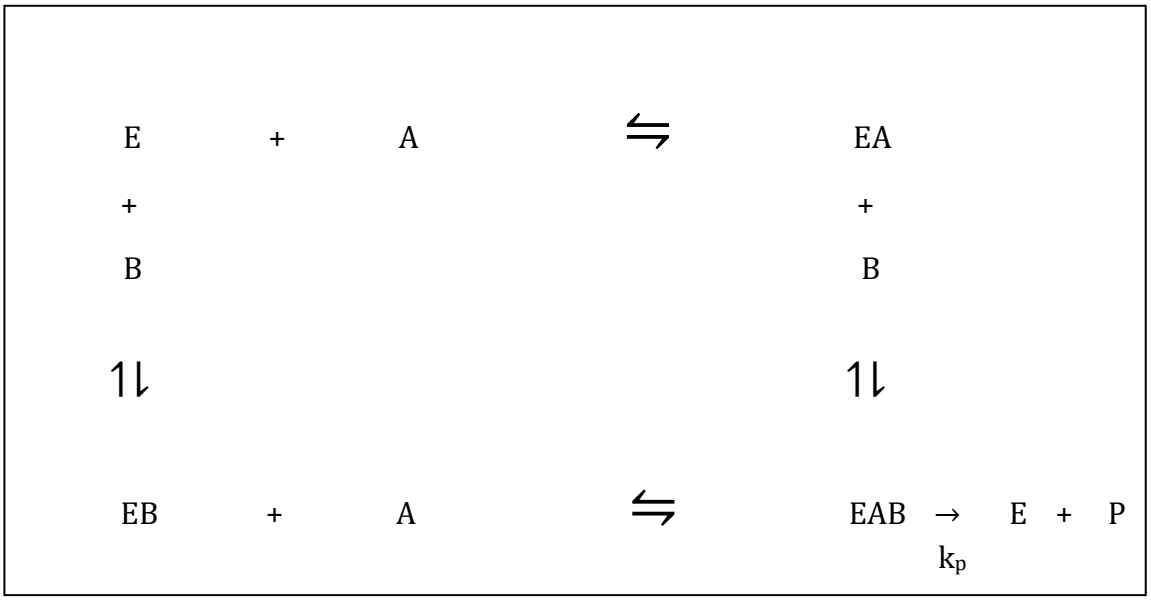

Figure 4. Random Sequential Binding. In the above figure random sequential binding is illustrated. The enzyme (E) binds to different substrates (A and B) in a random fashion irrespective of the sequence, which results in the formation of the EAB complex and finally yields the product P.

The enzyme systems EA, EB and EAB are called transitory complexes. These are defined as the complexes or enzyme species which can participate in a unimolecular reaction, or can isomerize to yield the products. A transitory system which cannot further participate in a unimolecular reaction but can successfully dissociate or isomerize to form product is called the central complex, in this case EAB.

Ordered Sequential Binding:

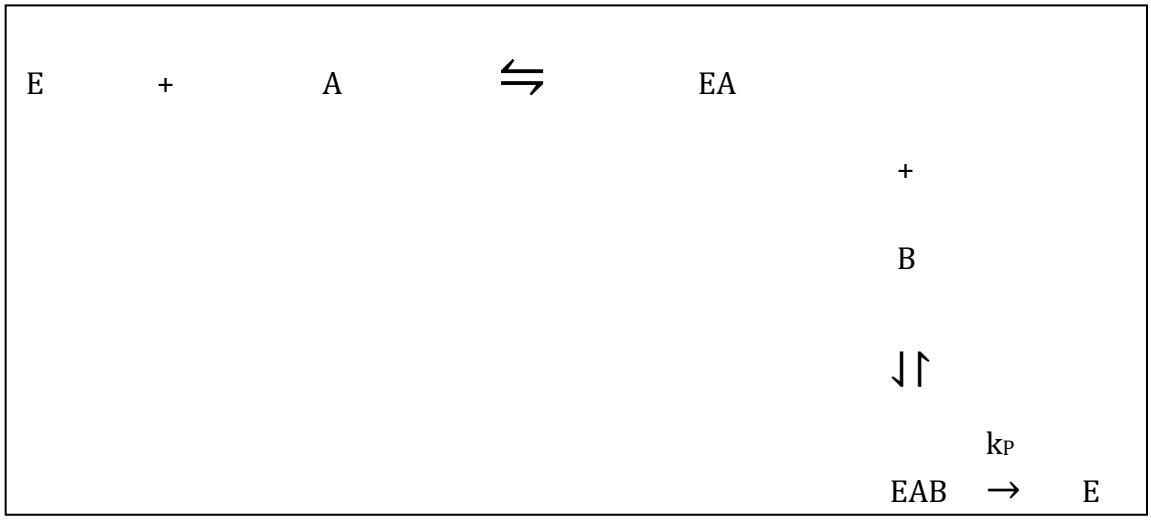

Figure 5. Ordered Sequential Binding. In the above figure ordered sequential binding is illustrated. Enzyme E binds to substrate A first and then to substrate B which results in the formation of the EAB complex and finally yields the product P.

The above equilibrium shows the ordered sequential system. In this system the activator A binds to only the free enzyme and the substrate B can bind EA complex but not the free enzyme.

Bisubstrate Enzyme Kinetic Models:

To understand the binding of cofactors Ca^{2+} and Cl^- to the enzyme and to understand if the reaction uses ordered sequential or random sequential binding the calculations are performed based on the data received from Lineweaver-Burk plots, specifically, plots of $\frac{1}{Ca^{2+}}$ vs y-intercepts of Lineweaver-Burk plots, and $\frac{1}{Ca^{2+}}$ vs. slopes of Lineweaver-Burk plots.

The following equation[31] applies to ordered sequential binding

$$\frac{1}{\mathrm{v}} = \frac{1}{[A]}\left(\frac{K^A{}_{\mathrm{S}}K^B{}_M}{\mathrm{V}_{max}} + \frac{K^A{}_{\mathrm{M}}}{\mathrm{V}_{max}[B]}\right) + \left(\frac{1}{V_{max}} + \frac{K^B{}_M}{V_{max}[B]}\right)$$

where,

$K_M{}^A$, $K_M{}^B$ are Michaelis constants for substrates A and B

$K_S{}^A$, $K_S{}^B$ are dissociation constants for substrates A and B

For random sequential binding the above equation is applicable, but the following version may also be used

$$\frac{1}{\mathrm{v}} = \frac{1}{[A]}\left(\frac{K^A{}_{\mathrm{S}}K^B{}_M}{\mathrm{V}_{max}\,K^B{}_S} + \frac{K^A{}_{\mathrm{S}}K^B{}_M}{\mathrm{V}_{max}[B]}\right) + \left(\frac{1}{V_{max}} + \frac{K^B{}_M}{V_{max}[B]}\right)$$

since for random sequential binding

$$K^{A}{}_{\mathrm{M}}K^{B}{}_{S} = K^{A}{}_{\mathrm{S}}K^{B}{}_{M}$$

This means that all four Michaelis/dissociation constants can be determined for random sequential binding, whereas ordered sequential binding has only three constants that can be determined.

Isothermal Titration Calorimetry:

Isothermal titration calorimetry (ITC) is a technique used for binding studies. It is used to study the thermodynamic parameters of a reaction or the binding of small molecules in a system. In the research described here, ITC was used to study the binding of primarily chloride in PSII.

There has been extensive study carried out to understand the binding constants of calcium and chloride in PSII. K_M values for activation by the ions were found out using the enzyme kinetic analysis in the experiments defined above. However, the use of ITC could also help in understanding the energetics involved in the binding of both calcium and chloride.

In ITC experiments heat absorbed or released during the endothermic or exothermic interactions respectively from the reference cell or to the reference cell is used to calculate the binding constant of the interaction. The ITC instrument is designed to have a reference cell, which is maintained at the same temperature as the cell containing the protein sample. The ligand is injected periodically in defined

amounts during the assay from the syringe into the protein cell. There is a certain amount of heat absorbed or released during each injection, which brings about temperature differences between the sample cell and the reference cell, which is then compensated by heating devices controlled by a feedback loop. To make sure the data is collected correctly, the concentration of the protein and the ligand must be adjusted in such a way that the amount of heat evolved or absorbed is measurable, in other words not too high or not too low. There must also be a differing (i.e., decreasing) heat change between each injection to give an efficient thermogram, which could can be used to obtain correct energetics. Each injection is collected as a peak on the thermogram, which is further used during the analysis to find out the number of binding sites or the stoichiometry of the interaction, the binding constant K_M and the heat associated with reaction ΔH. Analysis of this thermogram, which is a plot of heat change versus injection number, gives a sigmoidal curve, the shape of which depends upon the heat evolved or absorbed. The curvature depends on the Brandt's ''c'' parameter, which is a product of both the total molar concentration and the binding constant. A value of c = 50 gives a curvature that corresponds to good data for analysis. Making sure that the assay produces noise free data involves defined procedures for sample preparation, setting optimal concentrations for the samples and collecting the data.

EPR Spectroscopy:

An unpaired electron when placed in a strong magnetic field either aligns with its spin parallel to the magnetic field or spin antiparallel to the magnetic field. The difference in energy between the two spin states is proportional to the magnetic field strength and lifts the degeneracy of electron spin states normally present in the absence of a magnetic field. In electron paramagnetic resonance (EPR) spectroscopy,[32] a sample in a magnetic field is exposed to strong microwave radiation which excites the free unpaired electron from lower to higher energy states. As the magnetic field is swept, absorption of the microwave frequency matching the difference in higher and lower energy states is detected by the spectrometer. EPR spectroscopy is like NMR spectroscopy in many theoretical ways. However, the magnetic moment of electron spins exceeds that of proton spins by a factor of 660.

EPR spectroscopy studies are specific to very few compounds compared to NMR spectroscopy given the absence of unpaired electrons in many compounds compared to the presence of nuclei, in all the organic and inorganic compounds, required for NMR studies.[32]

During light excitation of the photosynthesis reaction, an electron is transferred from P680 to Quinone A (Q_A) and then to Q_B. An electron is then transferred from the Tyrosine Z molecule to the $P680^+$ radical. The Tyr Z radical is later reduced by the manganese cluster.[33] In the research conducted, here EPR

studies were performed to understand the behavior of the Tyr Z radical during this process of photosynthesis.

Research Overview:

Oxygen evolution assays were conducted using different concentrations of calcium and chloride after a special treatment of PSII for calcium depletion. Chloride and calcium dependence of PSII during oxygen evolution was studied using enzyme kinetics analysis of the ions as activators separately. Then bisubstrate enzyme kinetic analyses were carried out using these results.

To carry out chloride binding studies using isothermal titration calorimetry, different experiments were performed using different molar concentrations of NaCl-washed PSII lacking extrinsic subunits and chloride. Using the ITC data, attempts were made to calculate heats of the reaction, enthalpy and binding constants.

EPR spectroscopy was used to understand the Tyrosine Z signal in the absence of the calcium in the OEC of PSII. Tyrosine Z radical plays an important role in the transfer of electrons from the manganese cluster to P680 during photosynthesis and may be present as a radical when the Ca^{2+} is depleted. Since EPR detects unpaired electrons, it is expected to work as a great tool to assess the Tyr Z radical.

CHAPTER II

RESEARCH METHODS

Extraction of PSII:

Extraction of about 100-150 mg of PSII-enriched thylakoid membrane fragments from 600-800 grams of spinach was carried out. Earlier experiments by scientists usually employed thylakoid membranes, however Yocum and his group designed a procedure to extract active PSII using the detergent Triton X-100.[34] They confirmed the purity of the sample through activity and SDS-PAGE. This preparation showed no radical EPR signal due to P700+ of PSI, confirming the purity of the PSII extract.

In this preparation[34, 35], the spinach was blended and then filtered through four layers of nylon cheese cloth in buffer containing 350 mM sucrose, 20 mM MES and 10 mM NaCl at pH of 6.3. The sample was centrifuged at 5000 rpm in a JA10 Beckman rotor for 5 minutes. The pellet was suspended in buffer containing 20 mM MES-NaOH, 5 mM $MgCl_2.6H_2O$, 15 mM NaCl, pH 6.3 and centrifuged at 6500 rpm for 8 minutes. It was then resuspended to 2.5 mg of chlorophyll per mL (mgChl/mL) in the same buffer, followed by a dark incubation period of 60 –90 minutes. Triton X-100 was added to a final concentration of 5% and the suspension was incubated for another 30 minutes[34]. The sample was finally suspended and centrifuged several

times to remove the detergent in buffer containing 0.4 M sucrose, 20 mM MES, pH 6.3 and 15 mM NaCl. Finally, it was suspended in the same buffer to around 8-10 mgChl/mL and stored in liquid N_2. This preparation resulted in a PSII preparation with photosynthetically active oxygen evolving complex.

To carry out calcium and chloride binding studies with PSII, PsbP and PsbQ subunits are removed by incubation in high (1 – 2 M) NaCl concentration followed by centrifugation.[21] As described earlier the calcium and chloride ions are held in position with the help of PsbP and PsbQ subunits.[25, 28, 36] Removal of these subunits facilitates easy exchange of ions in their binding sites. The NaCl wash procedures bring down the activity to 30%-50% in the absence of calcium and almost to zero activity in the absence of chloride, and the residual activity is attributed to calcium ions which are not completely removed.

An incubation period of 30-35 minutes on ice in buffer with 1.5 M NaCl, 0.4 M sucrose and 20 mM MES at pH of 6.3 resulted in depletion of the two subunits.[21] Repetitive centrifugation of the sample in buffer without high NaCl concentrations at a speed of 15000 rpm in a Beckman JA 20 rotor was used to remove the excess NaCl and the released subunits. The PSII was resuspended in buffer and stored in liquid N_2. NaCl was omitted from wash buffers when the preparation was to be used for Cl^- dependence studies.

Additional Calcium Depletion Procedures:

Since a residual activity was observed even in the absence of calcium an additional treatment was used to remove calcium that remained bound. PSII samples were placed in buffer containing 1 mM EDTA before subjecting to illumination with actinic lights. This procedure helped in bringing down the activity to 20%.

The PSII was thawed and suspended to 1-1.5 mgChl/mL in 1000 μL of treatment buffer. The treatment buffer contained 1 mM EDTA, 20 mM NaCl, 0.4 M sucrose, and 50 mM MES-NaOH, pH 6.3. The sample was transferred onto ice and illuminated for 15 minutes using a Dolan-Jenner Fiber-Lite series 180 high intensity illuminator, set at low intensity of light, shaking the cryo tube every 5 minutes. The sample was then centrifuged at an rpm of 6000 in a microfuge for 2 minutes at a temperature of 4°C. The concentration of stock PSII was generally maintained at 1 mg/mL chlorophyll for almost all the assays of oxygen evolution.

O_2 Evolution Assays:

The amount of oxygen evolved was recorded using a Clark-type electrode (Yellow Springs Instrument #5331). In a usual Clark-type electrode setup, the electrode was placed in a sample chamber, which was enclosed in a water jacket. Within the electrode, current flows from platinum cathode to silver anode. In the chamber as the oxygen is evolved, the Pt cathode reduces O_2 to produce some form of water (H_2O or OH^-). As silver ions are left behind at the anode they combine with

chloride to form silver chloride. The higher the concentration of oxygen evolved in the chamber the greater is the current. The current is then converted to voltage and collected by the voltmeter, which is recorded by the program Logger Pro.

Overall reaction $4Ag + 4Cl^- + 4H^+ + O_2 \rightarrow 4AgCl + 2H_2O$

To calibrate the electrode, the amounts of oxygen in air-saturated water and in water purged with nitrogen (zero oxygen) were used. The slope of the voltage per time data thus obtained was calibrated to obtain the concentration of oxygen evolved per time.

To do the assays, the concentration of PSII was maintained at 0.02 mgChl/mL and different concentrations of NaCl (0, 2, 3, 5, 15, and 25 mM), with or without calcium, were used to check the chloride dependence of PSII. An electron acceptor, phenyl-*p*-benzoquinone (PPBQ) was added during assays at a concentration of 1 mM which is in great excess relative to PSII. An intact PSII extract produced 500-800 μmole O_2/mgChl/hr at 25^{0}C and pH 6.3.

ITC Titrations:

The ITC titrations were performed using an ITC200 microcalorimeter system (MicroCal, Inc.), with data fits carried out using the program MicroCal Origin. The ITC instrument has a protein sample cell, a reference buffer cell and also an injector for the ligand. The range of concentration used for the ligand, chloride ions, was from 0.2 mM to 25 mM. However, a later conclusion was derived that a higher

concentration of more than 4 mM might not provide reliable results. The buffer cell was filled with sucrose-MES buffer which was from dialysis of the PSII sample (see below). Maximum efforts were made to avoid any bubbles in any three of the chambers. The protein cell was filled with NaCl-washed with PSII. The data obtained during this experiment was analyzed and calibrated with the help of values such as the ligand and protein concentrations, expected K, number of injections, and volume of ligand for each injection.

To prepare for the ITC experiments the NaCl-washed PSII at a concentration of 1 mgChl/mL was further dialyzed to remove any leftover Cl^- ions. Also, the dialysis buffer was retained to use as the reference buffer solution, since it is very important to obtain the same buffer concentrations for the sample cell and the buffer cell to obtain noise free data. The NaCl-washed PSII was placed in dialysis bags (Spectra/Por, 12-14,000 molecular weight cutoff) in 2 mL aliquots and sealed. The dialysis bags were placed in 1 liter of buffer solution containing 0.4 M sucrose and 20 mM MES-NaOH, pH 6.3, at a temperature of 4 ^{0}C in the dark for overnight dialysis. Since there would have been left over Cl^- ions in the dialyzed buffer a second 2 hr dialysis was performed with same concentration of buffer. This gave dialyzed buffer free of Cl^- ions. After the completion of dialysis, the activity of PSII was recorded to make sure the Cl^- ions were removed.

EPR Experiments:

To prepare PSII samples for the EPR experiments, NaCl-washed PSII was split into four parts that were washed with 20 mM MES buffer, pH 6.3, containing different combinations of calcium and chloride. When calcium was present, the Ca^{2+} was added as $Ca(OH)_2$ before final adjustment of the pH was made with NaOH. The first sample contained neither calcium nor chloride. A second sample was prepared with 5 mM Ca^{2+} and no chloride. A third sample was prepared with no calcium and 20 mM NaCl. A fourth sample was prepared with 5 mM Ca^{2+} and 20 mM NaCl. These washes were performed using a Beckman JA 20 rotor at a speed of 15 k RPM and at a temperature 4^0C. Once these washes were performed the concentrations of the samples were checked and they were transferred to micro-centrifuge tubes after adjusting the volume to 1 ml with a concentration of 1 mgChl/mL.

PSII samples in 4 mm quartz EPR tubes were pre-illuminated for 5 s in an ice water bath at 0 ^{o}C, then dark-adapted on ice for 40-45 minutes before freezing in liquid nitrogen. The spectrum of the dark-adapted sample was then taken. Samples were thawed and illuminated for 20 s at 0 ^{o}C, then refrozen in liquid nitrogen. The spectrum of the illuminated sample was then taken. EPR spectroscopy was carried out using a Bruker Instruments model EMX 12/10 EPR spectrometer, equipped with a standard cavity. Samples were kept at 77 K using a liquid nitrogen EPR dewar. Instrument settings included microwave frequency at 9.45 GHz, microwave power at 1.0 mW, modulation frequency at 100 kHz, and modulation amplitude at 3 G.

CHAPTER III

RESEARCH DATA

Chloride and Calcium Dependence of O_2 Evolution Activity:

In this experiment, the dependence of oxygen evolution activity on chloride and calcium concentration was examined in NaCl-washed PSII, from which the PsbP and PsbQ subunits had been removed. The data were collected at a constant calcium concentration for a particular set of varying chloride concentrations. For every defined chloride/calcium concentration a triplet measurement of activity was made and averaged. The average activity in terms of μmole O_2/mgChl/hr was plotted against the chloride concentration.

The graph in Figure 6 represents the data collected for NaCl-washed PSII, additionally treated for calcium depletion, at calcium concentrations of 0.25, 0.5, 1, 3 and 5 mM, and Cl^- concentrations of 0, 2, 3, 5, 10 and 25 mM. The data were plotted with the help of SigmaPlot, which was used to perform fits to find the V_{max} and K_M values for the individual curves.

The graph in Figure 7 represents a cumulative Lineweaver-Burk plot. The graph is a plot between the reciprocal of the chloride concentration and the reciprocal of the oxygen evolution activity. The five different plots are for the five different concentrations of calcium.

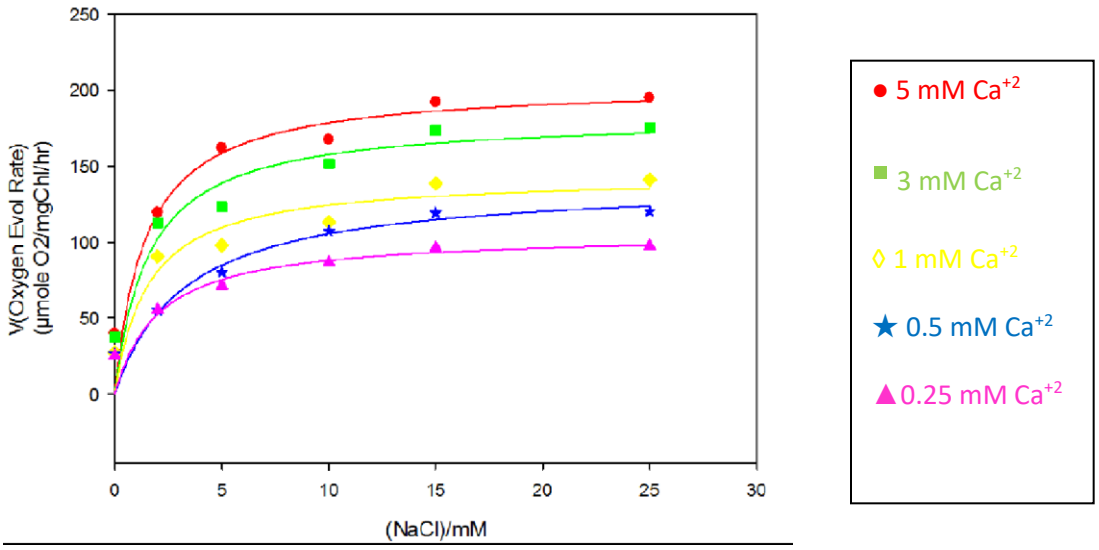

Figure 6. Oxygen Evolution Activity of Calcium Depleted NaCl-washed PSII. This was conducted with five different calcium concentrations (5, 3, 1, 0.5 and 0.25 mM) and varying chloride concentrations (0, 3, 5, 15 and 25 mM) at pH 6.3. Each point is the average of three values.

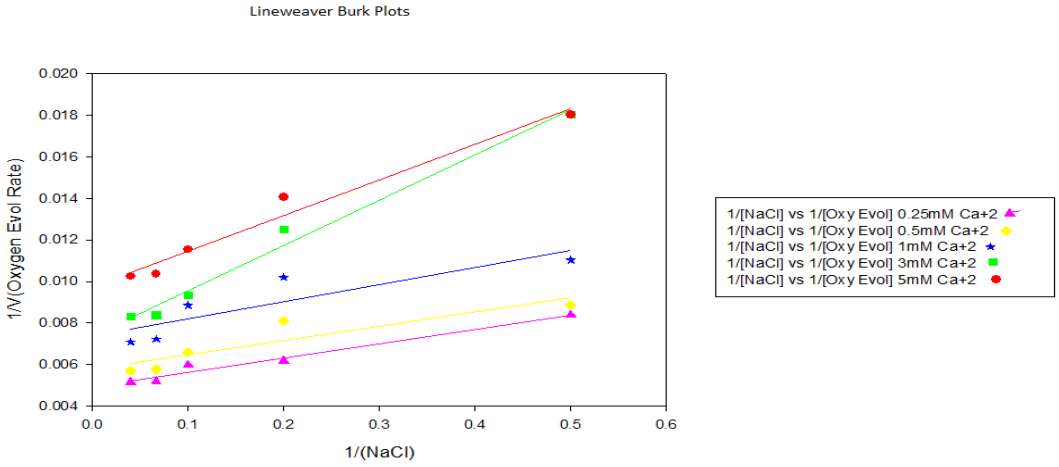

Figure 7. Lineweaver-Burk Plots for the Activation Curves shown in Figure 6.

Kinetic analysis of the oxygen evolution data provided apparent V_{max} and K_M values for the various calcium concentrations (Table 2). Direct fits to the Michaelis-Menten equation, which required a non-linear regression, were used to analyze the kinetic data. Lineweaver-Burk plots, 1/v vs 1/[S], are linear plots of the same data, so linear regressions were used to analyze those plots. However, the K_M and V_{max} values obtained from the Lineweaver-Burk plots are not as reliable since reciprocal values of the data result in large error for some points. Hence the direct fits to the Michaelis-Menten curves are preferred over Lineweaver-Burk fits. The Lineweaver-Burk plots however give a view of the type of inhibition the enzyme could be participating in.

V_{max} AND K_M Values:

Table 2. V_{max} and K_M Values for Chloride Activation of Oxygen Evolution at Various Calcium Concentrations. The second set of V_{max} and values (designated LB) were obtained from the Lineweaver-Burk plots based on the y-intercepts and the slopes.

Calcium Conc	0.25 mM	0.5 mM	1 mM	3 mM	5 mM
V_{max} / $\mu moleO_2$ $mgChl^{-1}$ hr^{-1}	106 ± 13	140 ± 17	144 ± 16	182 ± 20	204 ± 19
K_M / mM	2.0 ± 1.2	3.2 ± 1.5	1.6 ± 0.9	1.6 ± 1	1.4 ± 0.7
V_{max}(LB) / $\mu moleO_2$ $mgChl^{-1}$ hr^{-1}	103 ± 4	135 ± 7	135 ± 12	174 ± 14	203 ± 7
K_M (LB) / mM	1.8 ± 0.2	2.9 ± 0.2	1.1 ± 0.4	1.2 ± 0.1	1.4 ± 0.1

It can be seen from the data in the above Table 2, the V_{max} values increased with increasing calcium concentrations, however a trend was not observed for K_M values as the values increased from 0.25 mM to 0.5 mM calcium concentrations and then there was a decrease from 0.5 mM to 1 mM and 3 mM to 5 mM calcium concentrations.

Bisubstrate Enzyme Kinetics Analysis:

The next step in the bisubstrate analysis of the calcium and chloride ions involved the secondary plots. The graphs in Figure 8 represent the secondary plots between the y-intercept and inverse of calcium concentration and the plots between slopes and inverse of calcium concentration.

Secondary Plots:

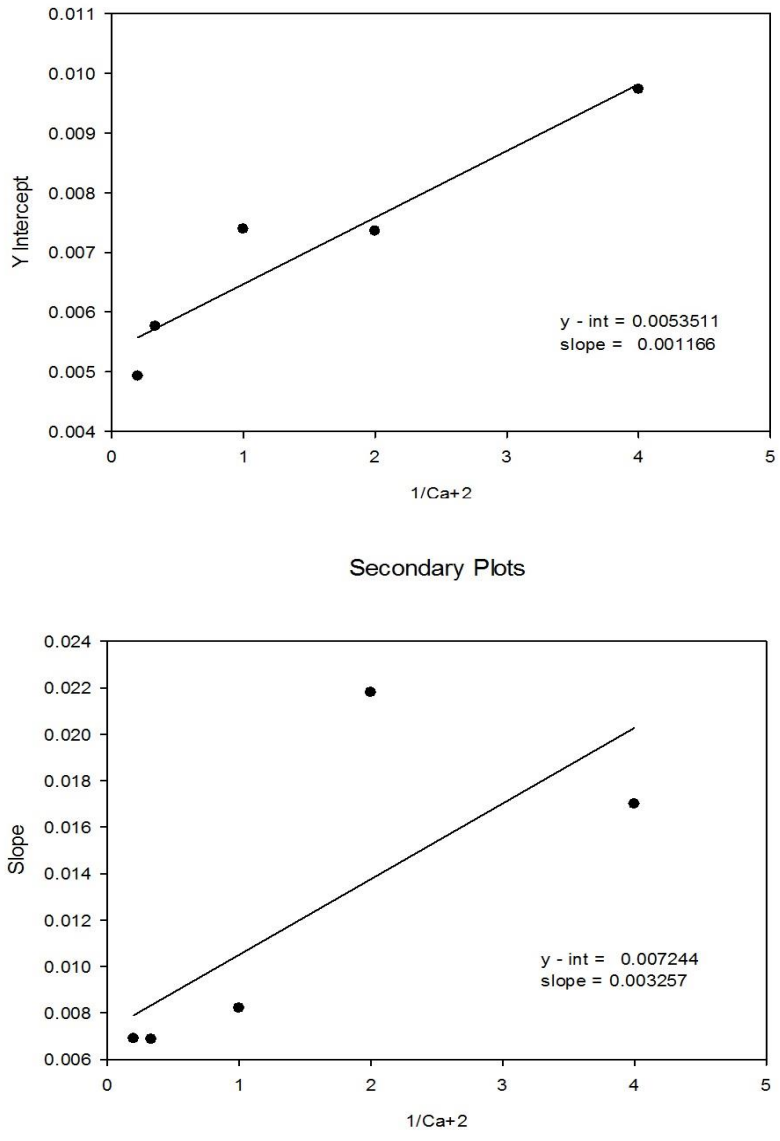

Figure 8. Secondary Plots for Bisubstrate Analysis. Based on the data in Figure 7. Top: Lineweaver-Burk y-intercepts versus the inverse of calcium concentration; bottom: Lineweaver-Burk slopes versus the inverse of calcium concentration.

Bisubstrate enzyme kinetics analysis helps us understand the binding mechanism followed by the enzyme PSII and the substrates Ca^{2+} and Cl^-. Analysis of the oxygen evolution activity using random and ordered sequential binding models produced Michaelis constants for both calcium and chloride and also a dissociation constant for chloride. The dissociation constant for calcium could be obtained only from the random sequential binding model.

The following equations are those used to carry out the bisubstrate analysis for both the ordered and random sequential model. In this analysis, A and B = Cl^- and Ca^{2+}. The K_M values remain the same irrespective of A and B being calcium or chloride.

$$\frac{K^A{}_M}{V_{max}} + \frac{K^A{}_S K^B{}_M}{V_{max}[B]} = slope\ of\ Lineweaver - Burk\ plots$$

$$\frac{1}{V_{max}} + \frac{K^B{}_M}{V_{max}[B]} = y - intercept\ of Lineweaver - Burk\ plots$$

From plots of $\frac{1}{Ca^{2+}}$ vs y-intercepts of Lineweaver–Burk plots:

$$\frac{1}{V_{max}} = \text{secondary y–intercept}$$

$$\frac{K^B{}_M}{V_{max}} = \text{secondary slope}$$

From plots of $\frac{1}{Ca^{2+}}$ vs slopes of Lineweaver–Burk plots:

$$\frac{K^{A}{}_{S}K^{B}{}_{M}}{V_{max}} = \text{secondary slope}$$

$$\frac{K^{A}{}_{M}}{V_{max}} = \text{secondary y–intercept}$$

For random sequential binding, the value of $K^{B}{}_{S}$ can be determined through the relationship

$$K^{A}{}_{M}K^{B}{}_{S} = K^{A}{}_{S}K^{B}{}_{M}$$

The results of the analysis are shown in Table 3.

Table 3. Michaelis Constants and Dissociation Constants Obtained from Bisubstrate Enzyme Kinetic Analysis

Parameter	Values from ordered and random binding model
V_{max}	187 ± 15 μmole O_2/mgChl/hr
$K^{Ca^{2+}}{}_{M}$	0.21 ± 0.04 mM
$K^{Ca^{2+}}{}_{S}$	0.45 ± 0.32 mM (from random binding model)
$K^{Cl^-}{}_{S}$	2.9 ± 1.6 mM
$K^{Cl^-}{}_{M}$	1.3 ± 0.7 mM

Conclusions for Enzyme Kinetic Studies:

In this experiment an additional depletion procedure was performed to remove any residual calcium that might be present in NaCl-washed PSII. Although not all the calcium and chloride were removed from the PSII by NaCl-washing alone, as it can be seen from the experimental data that the PSII had activity even with no calcium and chloride added, activity was reduced to less than 20% by performing the additional calcium depletion procedure. After the depletion procedures and removal of calcium and chloride the activity of the PSII was restored by re-addition of Ca^{2+} and Cl^{-}, allowing determination of enzyme kinetic parameters for activation by these ions.

An effort was made to understand the bisubstrate enzyme activity in PSII. From the data obtained here, though, it cannot be distinguished whether the binding of the two activators is random or ordered. The K_M and K_S values of chloride are higher than those of calcium, indicating the higher affinity of calcium at the OEC during the oxygen evolution process. In addition, the K_M and K_S values of chloride from the bisubstrate analysis (1.3 and 2.8 mM) are within the range of values for K_M found from the direct fits to the individual activation curves (1.1 to 2.9 mM, Table 2). This would be expected if there is no influence of calcium on chloride activation other than to provide an active enzyme.

Isothermal Titration Calorimetry:

A part of the goal of this project includes binding studies of calcium or chloride ions to PSII with the help of isothermal titration calorimetry (ITC). Literature is available on the binding of various systems with calcium and their study using isothermal titration calorimetry. For example, a study was carried out by Yi-Pin Lin and his group on the binding of calcium to Leptospiral immunoglobulin-like protein using ITC.[37] The dissociation constant for the binding of calcium to the protein was calculated as 7 μM.

A frequently used test for ITC is the binding of Ca^{2+} to EDTA. The following titration (Figure 9) was conducted between EDTA and $CaCl_2$ in 20 mM MES buffer at pH 6.3. The ITC cell held 250 μL of protein and the syringe held 40 μL of ligand, which was spread across 15 injections (2.7 μL per injection). EDTA was present at a concentration of 0.5 mM in the ITC cell and $CaCl_2$ was used as the ligand in the syringe at a concentration of 50 mM. Both ligand and the protein were prepared in 20 mM MES buffer. The temperature was maintained at 25^0C. The binding constant was calculated to be 2.78×10^{-7} M with an error margin of 1.48×10^{-4} M. The data clearly showed the presence of one binding site.

Calcium and EDTA ITC Assay:

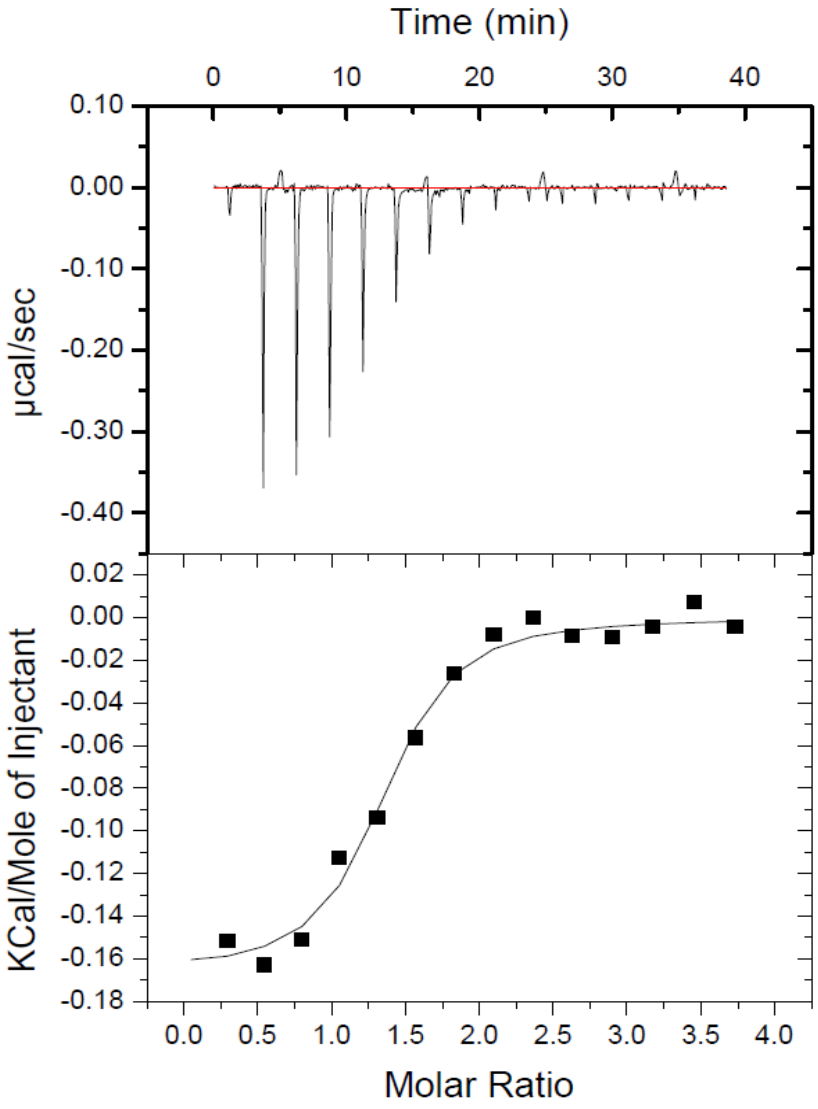

Figure 9. Data Collected from an ITC Run of EDTA and $CaCl_2$. The top panel illustrates the heat changes observed over a series of 15 injections of Ca^{2+} into the EDTA solution. The lower panel shows the heats evolved as a quantitative curve of Ca^{2+} binding to EDTA.

An effort was made to use ITC to study the binding of chloride to PSII, although the affinity of PSII for chloride is much lower than the typical affinity of calcium-binding proteins for calcium. NaCl-washed PSII, which lacked PsbP and PsbQ subunits, were used to carry out the binding studies. Samples were prepared by dialysis against chloride–free buffer, with 0.4 M sucrose, 20 mM MES, pH adjusted to 6.3 with 5 mM $Ca(OH)_2$ and NaOH. NaCl-washed PSII was dialyzed twice, consecutively, where the initial dialysis was conducted for 8 hours and the secondary dialysis for 3 hours. The buffer from the dialysis was used for the reference buffer in ITC experiments, to ensure that it was the same as the sample buffer. The PSII sample concentration was maintained at 1 mgChl/ml for most of the ITC experiments.

To carry out the binding studies of chloride with PSII, different procedures were used by trial and error, including different molar ratios of NaCl and PSII. Initial preliminary data was collected using higher concentrations of NaCl-washed PSII. However, at higher PSII concentrations, the heats of reaction were very high for the ITC to read data. The sensitivity of ITC was comparatively very low when compared to the binding energy released during the binding of chloride. Hence an effort was made to increase the volume of the sample without increasing the PSII concentration, with the following data obtained.

Figure 10 shows an experiment conducted in which aliquots of 200 μL of 100 μM stock NaCl was added to 40 μL of 4.4 μM NaCl-washed PSII in the sample cell. This experiment was carried out in collaboration with Prof. Maria Ngu-Schwemlein

at the Department of Chemistry, Winston-Salem State University, using an ITC instrument that was like the MicroCal ITC200 but with a larger sample cell than the one in the Department of Chemistry and Biochemistry at UNCG. The trace suggests both endothermic and exothermic heat exchanges, but they were very small in general. Overall it was not possible to determine whether the heats of interaction were due to binding or to some other non-specific interaction (e.g., conformational changes).

PSII and NaCl ITC Assay:

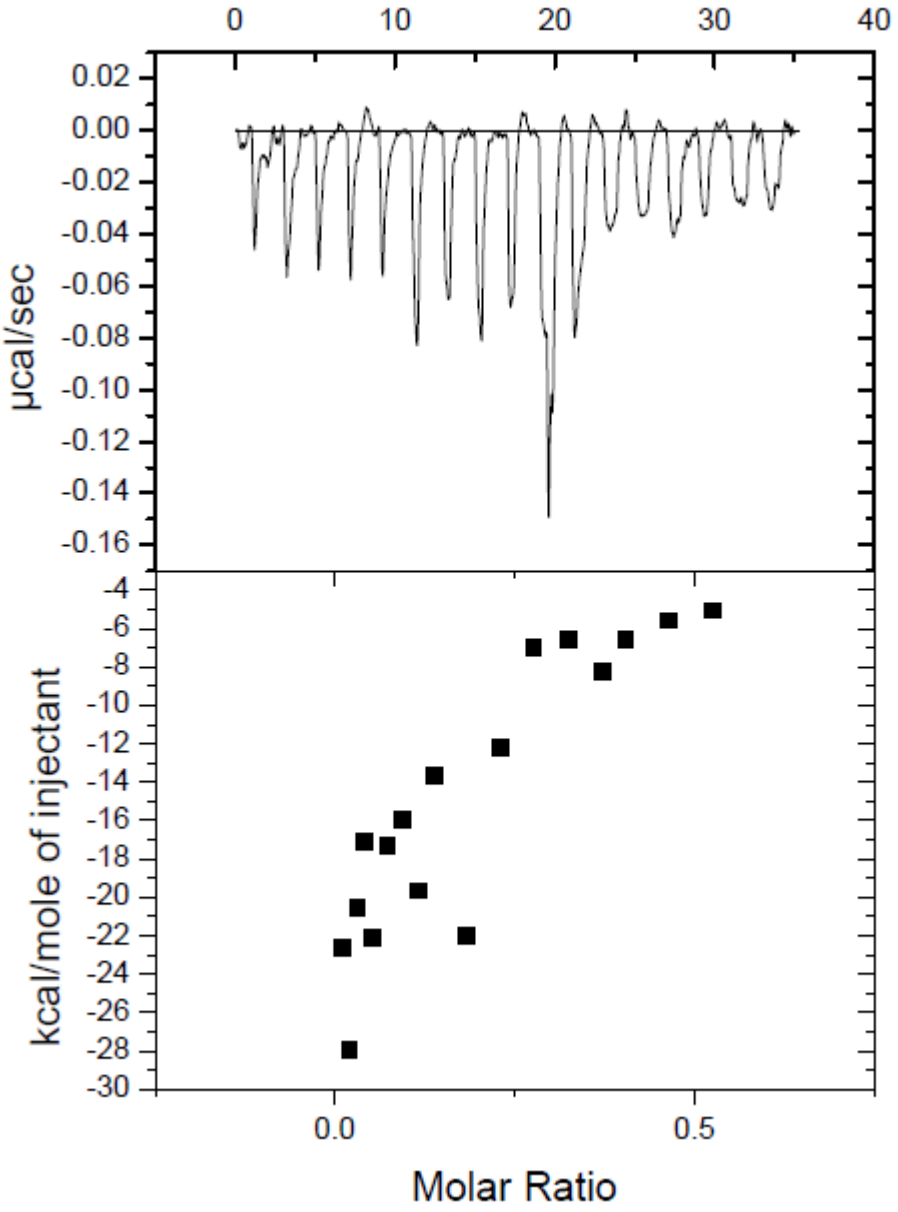

Figure 10. ITC Data for the Binding of NaCl to PSII. The syringe contained 200 µL of 100 µM stock NaCl, which was added to 40 µL of 4.4 µM NaCl-washed PSII in the sample cell. 18 injections were made.

Conclusions for ITC Study of Chloride Binding:

The high sensitivity of the ITC technique was hoped to provide accurate binding analysis from the PSII system. In order to calibrate the data obtained during the ITC experiments, large heat changes should be obtained at regular intervals, with heat changes decreasing after which saturation is observed. With the PSII system this synchrony of the data was absent. Two reasons could account for this: either the ions were attaining saturation with very few injections of the ligand or the heats evolved were too low for the technique to record. After multiple experiments were conducted using the ITC for studies of chloride binding to PSII, it was concluded that these studies were not able to yield good data given the difficulty in handling higher concentrations of PSII combined with the lower capacity of the ITC sample cell.

It was thought that the experiments could be better performed if a bigger cell for the protein sample was used and more promising data was indeed obtained in one or two test experiments using an ITC instrument at Winston-Salem State University, with the help of Dr. Ngu-Schwemlein. Although heat exchanges were observed over many injections, this may have corresponded to non-specific binding or general conformational changes related to ionic strength, thereby obscuring the specific chloride binding that may have taken place. Unfortunately, these experiments could not be continued because of problems with the instrument.

Electron Paramagnetic Resonance (EPR) Spectroscopy:

Theoretically, when PSII contains both calcium and chloride ions the transfer of electrons takes place in very quick succession, thus the Tyr Z radical EPR signal recorded for the sample with calcium and chloride should be weaker than for the sample without both these ions. This is because if the electron transfer chain is incomplete, such as when ion cofactors are missing, the Tyr Z radical is unable to be reduced by the Mn_4Ca cluster. PSII samples containing only added calcium may be expected to show a stronger Tyr Z signal than PSII with only added chloride, because residual calcium is more likely to be present in the latter samples.

PSII subunits PsbP and PsbQ were removed by high concentration NaCl treatment, followed by washing in buffer containing 20 mM MES and 0.4 M sucrose. The final wash buffer was varied to obtain four different NaCl washed PSII samples which contained either calcium and chloride, calcium alone, chloride alone, or neither ion (Table 4). The Tyr radical EPR signal was measured at 77 K in each sample after dark-adaptation on ice and again after illumination of the sample for 20 s at 0 ^{o}C. The signal in the dark-adapted sample represents Tyr D, the dark-stable tyrosine radical, whereas the signal in the illuminated sample represents both Tyr D and Tyr Z (Figure 11).

Tyrosine Z in Photosystem II:

Table 4. EPR of PSII Samples. Tyr radical EPR signal heights in NaCl-washed PSII samples prepared with various chloride and calcium concentrations. The heights of the Tyr signal in the dark- adapted sample and the sample after illumination were measured from peak to trough and are given in arbitrary relative units. The last column shows the percent increase in the signal after illumination compared to the dark-adapted signal.

Sample Name	**[Cl^-] / mM**	**[Ca^{2+}] / mM**	**Tyr EPR signal, Dark-adapted**	**Tyr EPR signal, Illuminated**	**% Tyr Z**
Sample 1	0	0	78337	113606	45%
Sample 2	20	0	87238	96421	10%
Sample 3	0	5	79322	109505	38%
Sample 4	20	5	73591	104175	41%

EPR Spectroscopy Samples:

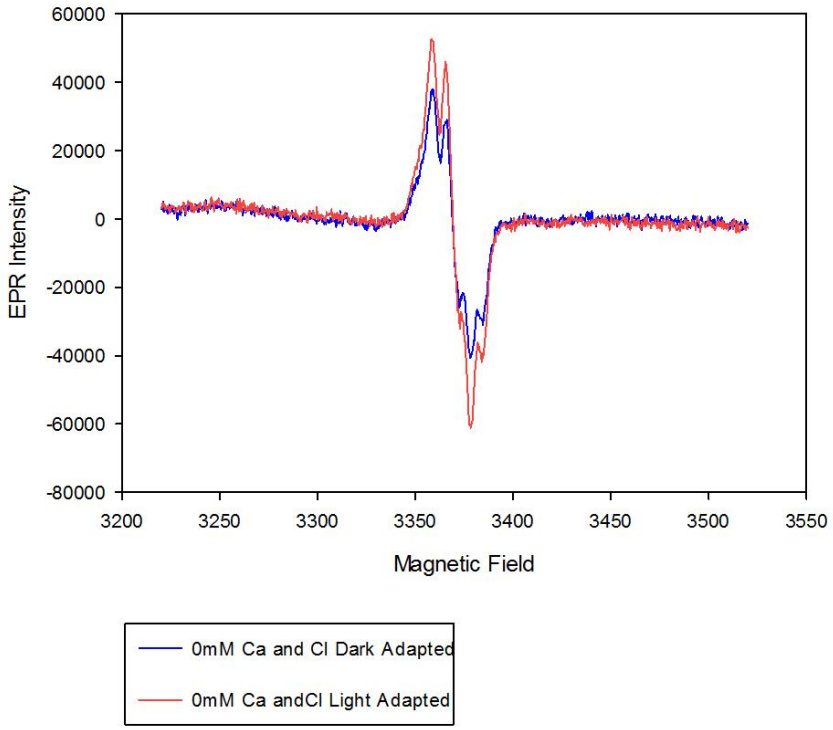

Figure 11. EPR Spectroscopic Study for 0 mM Ca^{+2} and Cl^{-}. The above data is for dark adapted and illuminated PSII samples.

Conclusions for EPR Spectroscopy:

It was expected that the PSII sample lacking both chloride and calcium would show a high amount of Tyr Z and this was found to be so, with 45% Tyr Z signal. However, the sample containing both calcium and chloride was expected to show the least signal, but this sample showed almost as much Tyr Z at 41%. The sample showing the least Tyr Z signal was found to be the one containing chloride only, with

only 10% signal,while the sample containing calcium only showed a fairly large amount of Tyr Z signal at 38%, as is expected if calcium alone is insufficient to promote electron transfer.

If the samples were correctly identified, this result suggests that the addition of chloride alone could restore electron transfer; this would be the case if calcium remained bound to the Mn_4Ca cluster during the preparation of the samples. Although the treatment was intended to remove the Ca^{2+}, it is well known that it is more difficult to remove Ca^{2+} from PSII than to remove Cl^-. In addition, the results suggest that the presence of added calcium suppressed activity, perhaps through an unknown effect of excess calcium.

CHAPTER IV

RESEARCH ACCOMPLISHMENTS

The following results were obtained from the thesis project.

i) Measurements of oxygen evolution activity using varying concentrations of calcium and chloride were done using NaCl-washed PSII, which lacked the extrinsic PsbP and PsbQ subunits. Better understanding of the calcium and chloride requirements was achieved by conducting oxygen evolution assays at concentrations of calcium ranging from 0.25 mM to 5 mM and chloride concentrations ranging from 0 mM to 25 mM. Analysis of the data collected from these experiments confirmed that both calcium and chloride were required for activity and showed that the oxygen evolution activity increased as the concentration of chloride and/or calcium increased. The K_M and V_{max} values for chloride activation were collected with the help of Michaelis-Menten curves. It could be seen that with the increasing calcium concentrations the V_{max} values increased, however the K_M values for chloride activation were within error of each other.

ii) Analysis of Ca^{2+} and Cl^- activation of oxygen evolution in terms of bisubstrate enzyme kinetics was done, by application of the kinetic

iii) equations for random and sequential ordered binding. The analysis revealed the K_M and K_S values for calcium and chloride binding that are

iv) intrinsic to the sites of activation. However, the order of binding could not be determined from this analysis.

v) Isothermal titration calorimetry (ITC) was used for characterization of the binding of chloride to PSII, with the main objective of assessing whether the method can be useful for this system. Assays were conducted with NaCl-washed PSII samples. Since the data were not conclusive we could not identify the binding stoichiometry, the binding constants and the heats involved in the interaction. The data did not show clear sigmoidal fitting and this resulted in inaccurate binding energies. Although adjustments were made in the experimental conditions, the data did not show a desirable result since the binding affinity was not high enough for ITC to read the data.

vi) Initial EPR spectroscopy studies were carried out to understand the response of the Tyr Z radical to the presence of calcium and/or chloride. The presence of the Tyr Z radical indicates inhibition of electron transfer activity. The experiments were also intended to assess whether the method could be used to determine the order of binding of calcium and chloride. The results suggested that the addition of chloride alone restored the ability of PSII to transfer of electrons.

REFERENCES

1. Fromme, P.; Yu, H.; Yana, S. D.; Jolley, C.; Chauhan, D.K.; Melkozernov, A.; Grotjohann, I., Full paper / Mémoire: Structure of photosystems I and II. *Comptes rendus - Chimie* 2006, *9*, 188-200.
2. Govindjee, G.; Govindjee, R., The Absorption of Light in Photosynthesis. *Scientific American* 1974, *231* (6), 68-86.
3. Hall, D. O.; Rao, K. K., *Photosynthesis / D.O. Hall and K.K. Rao*. Cambridge; New York: Cambridge University Press, 1999.
4. Renger, G.; Renger, T., Photosystem II: The machinery of photosynthetic water splitting. *Photosynthesis Research* 2008, *98* (1-3), 53-80.
5. Vrettos, J. S.; Brudvig, G. W., Water Oxidation Chemistry of Photosystem II. *Philosophical Transactions: Biological Sciences* 2002, *357* (1426), 1395-1405.
6. McConnell, I. L., Substrate water binding and oxidation in photosystem II. *Photosynthesis Research* 2008, *98* (1-3), 261-276.
7. Zouni, A.; Witt, H. T.; Kern, J.; Fromme, P.; Krauss, N.; Saenger, W.; Orth, P., Crystal structure of photosystem II from Synechococcus elongatus at 3.8 angstrom resolution. *Nature* 2001, *409* (6821), 739-743.
8. Guskov, A.; Kern, J.; Gabdulkhakov, A.; Broser, M.; Zouni, A.; Saenger, W., Cyanobacterial photosystem II at 2.9 Å resolution and the role of quinones, lipids, channels and chloride. *Nature Structural & Molecular Biology* 2009, *16* (3), 334-342.
9. Umena, Y.; Kawakami, K.; Shen, J.-R.; Kamiya, N., Crystal structure of oxygen-evolving photosystem II at a resolution of 1.9 Å. *Nature* 2011, *473* (7345), 55-60.
10. Klein, M.; Sauer, K.; Yachandra, V., Perspectives on the structure of the photosynthetic oxygen evolving manganese complex and its relation to the Kok cycle. *Photosynthesis Research* 1993, *38* (3), 265.
11. Roelofs, T. A.; Liang, W.; Latimer, M. J.; Cinco, R. M.; Rompel, A.; Andrews, J. C.; Sauer, K.; Yachandra, V. K.; Klein, M. P., Oxidation States of the Manganese Cluster during the Flash-Induced S-State Cycle of the Photosynthetic Oxygen-Evolving Complex. *Proceedings of the National Academy of Sciences of the United States of America* 1996, *93* (8), 3335-3340.
12. Bridgette A. Barry; Ian B. Cooper; Antonio De Riso; Scott H. Brewer; Dung M. Vu; R. Brian Dyer; Time-resolved vibrational spectroscopy detects protein-based intermediates in the photosynthetic oxygen-evolving cycle. *PNAS* 2006, 103 (19), 7288–7291.

13. Kern, J.; Biesiadka, J.; Loll, B.; Saenger, W.; Zouni, A., Structure of the Mn4–Ca cluster as derived from X-ray diffraction. *Photosynthesis Research 2007,* 92(3), 389-405.
14. Yano, J.; Kern, J.; Pushkar, J.; Sauer, K.; Glatzel, P., Bergmann, U.; Messinger, J.; Zouni, A.; Yachandra, V. K., High-resolution structure of the photosynthetic Mn4Ca catalyst from X-ray spectroscopy. *Philos Trans R Soc Lond B Biol Sci 2008,* 363(1494),1139-47
15. Limburg, J.; Vrettos, J.S.; Liable-Sands, L.M.; Rhingold, A.L.; Crabtree, R.H.; Brudvig, G.W., A functional model for O-O bond formation by the O_2-evolving complex in photosystem II. *Science* 1999, 283 (5407)
16. Sandusky, P. O.; Yocum, C. F., Regular paper: The chloride requirement for photosynthetic oxygen evolution: Factors affecting nucleophilic displacement of chloride from the oxygen-evolving complex. *BBA - Bioenergetics* 1986, *849*, 85-93.
17. Siegbahn, PE., Mechanism and energy diagram for O-O bond formation in the oxygen-evolving complex in photosystem II. *Philos Trans R Soc Lond B Biol Sci*. 2008, 363(1494), 1221-8
18. Wincenjusz, H.; Van Gorkom, H. J.; Yocum, C. F., The photosynthetic oxygen evolving complex requires chloride for its redox state S2-->S3 and S3-->S0 transitions but not for S0-->S1 or S1-->S2 transitions. *Biochemistry 1997*, 36(12),3663-70.
19. Grove, G. N.; Brudwig, G. W., Calcium binding studies of photosystem II using a calcium-selective electrode. *Biochemistry* 1998, *37* (6), 1532.
20. Adelroth, P.; Lindberg, K., Studies of Ca^{2+} binding in spinach photosystem II using $^{45}Ca^{2+}$. *Biochemistry* 1995, *34* (28), 9021.
21. CM Waggoner, V Pecoraro, CF Yocum., Monovalent cations (Na+, K+, Cs+) inhibit calcium activation of photosynthetic oxygen evolution. *FEBS Letters* 1989, *244*, 237-240.
22. Haddy, A.; Ore, B. M., An Alternative Method for Calcium Depletion of the Oxygen Evolving Complex of Photosystem II As Revealed by the Dark-Stable Multiline EPR Signal. *Biochemistry, 2010*, 49 (18), 3805–3814.
23. Han, K. C. and Katosh, S.; Different Localization of Two Ca^{2+} in Spinach Oxygen-Evolving Photosystem II Membranes. Evidence for Involvement of Only One Ca^{2+} in Oxygen Evolution.*Oxford Journals* 1993, 34(4), 585-593.
24. Miqyass, M.; van Gorkom, H. J.; Yocum, C. F., The PSII calcium site revisited. *Photosynthesis Research Springerlink* 2007, *92* (3), 275-287.
25. Cox, N., Rapatskiy, L., Su J.H, et.al; Effect of Ca^{2+}/Sr^{2+} Substitution on the Electronic Structure of the Oxygen-Evolving Complex of Photosystem II: A Combined Multifrequency EPR,^{55}Mn-ENDOR, and DFT Study of the S_2 State. *JACS* 2011, 133(10), 3635–3648.
26. Vrettos, J. S.; Stone, D. A.; Brudvig, G. W., Quantifying the ion selectivity of the Ca2+ site in photosystem II: Evidence for direct involvement of Ca2+ in O-2 formation. *Biochemistry* 2001, *40* (26), 7937-7945.

27. Pokhrel, R.; McConnell, I. L.; Brudvig, G. W., Chloride Regulation of Enzyme Turnover: Application to the Role of Chloride in Photosystem II. *Biochemistry* 2011, *50* (14), 2725-2734.
28. Lindberg, K.; Vanngard, T.; Andreasson, L-E., Studies of the slowly exchanging chloride in photosystem II of higher plants. *Photosynth Res 1993,* 38, 401–408
29. Haumann, M.; Barra, M.; Loja, P.; Löscher, S.; Krivanek, R.; Grundmeier, A.; Andreasson, L.-E.; Dau, H., Bromide does not bind to the Mn4Ca complex in its S1 state in Cl^--depleted and Br^--reconstituted oxygen-evolving photosystem II: evidence from X-ray absorption spectroscopy at the Br K-edge. *Biochemistry* 2006, *45* (43), 13101-13107.
30. Bryson, D. I.; Doctor, N.; Johnson, R.; Baranov, S.; Haddy, A, Characteristics of Iodide Activation and Inhibition of Oxygen Evolution by Photosystem II. *Biochemistry 2005, 44* (19), 7354–7360
31. Schnell, S.; Maini, P.K.; Enzyme Kinetics at High Enzyme Concentration. *Bulletin of Mathematical Biology* 2000, 62, 483–499
32. Rana, S.; Chawla, R; Kumar, R; et.al; Electron paramagnetic resonance spectroscopy in radiation research: Current status and perspectives. *J Pharm Bioallied Science* 2010, 2(2), 80–87.
33. Ayala, I; Kim, S; Barry, B.B; A Difference Fourier Transform Infrared Study of Tyrosyl Radical Z Decay in Photosystem II. *Biophysical Journal* 1999, 77, 2137–2144
34. Berthold, D. A., Babcock, G. T., and Yocum, C. F., A highly resolved, oxygen-evolving photosystem II preparation from spinach thylakoid membranes. EPR and electron-transport properties. *FEBS Letters* 1981, *134*, 231-234.
35. Ford, R. C.; Evans, M. C. W., Isolation of a photosystem 2 preparation from higher plants with highly enriched oxygen evolution activity. *FEBS Letters* 1983, *160*, 159-164.
36. Popelková, H.; Yocum, C. F., Current status of the role of Cl^- ion in the oxygen-evolving complex. *Photosynthesis Research* 2007, *93* (1-3), 111-121.
37. Lin, Y-P.; Raman, R; Sharma, Y; and Chang, Y-F; Calcium Binds to Leptospiral Immunoglobulin-like Protein, LigB, and Modulates Fibronectin Binding. The Journal of Biological Chemistry 2008, 283(37), 25140-25149.